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Brief Report

Increased yield of porcine circovirus-2 by a combined treatment of PK-15 cells with interferon-gamma and inhibitors of endosomal-lysosomal system acidification

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Summary

Treatment of porcine kidney (PK-15) cells with either interferon-gamma (IFN-γ) or endosomal-lysosomal system acidification inhibitors increases replication of porcine circovirus type 2 (PCV2). In the present study, the effect of a combination of these treatments on the number of infected cells and virus yield was tested. The number of PCV2 (Stoon-1010)-infected PK-15 cells increased in cells treated with ammonium chloride ($445 \pm 39\%$ increase), IFN-γ ($446 \pm 8\%$), ammonium chloride + IFN-γ ($1721 \pm 283\%$), chloroquine diphosphate ($1037 \pm 121\%$), chloroquine diphosphate + IFN-γ ($2199 \pm 255\%$), monensin ($950 \pm 178\%$) and monensin + IFN-γ ($1948 \pm 60\%$). Combined IFN-γ and endosomal-lysosomal system acidification inhibitors increased PCV2 yield by up to 50 times compared to untreated PK-15. This augmented virus replication in PK-15 cells may be helpful in the production of PCV2 vaccines.

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Porcine circovirus type 2 (PCV2) is a member of the genus *Circovirus*, family *Circoviridae* [30]. PCV2 infections are mainly associated with a multifactorial disease in pigs, postweaning multisystemic wasting syndrome (PMWS) [1, 7, 9, 12, 17, 27]. Apart from PMWS, PCV2 has also been associated with various disease syndromes in pigs including porcine dermatitis and nephropathy syndrome [3, 5, 19, 21, 31, 35, 37], congenital tremors [6, 14], reproductive failure [16, 18, 21, 28, 29, 33], exudative epidermitis [38], porcine respiratory disease complex [13], proliferative and necrotizing pneumonia [11] and enteritis [4, 15]. *In vitro*, PCV2 replicates in porcine kidney epithelial cells [2, 20, 25, 32], and PCV2 isolation and production is routinely performed using porcine kidney (PK-15) and swine kidney (SK) epithelial cell lines [2, 32]. When PCV2 replication kinetics were studied in a PK-15 epithelial cell line that was inoculated with the prototype PCV2 strain Stoon-1010 [9], 1.8% of cells were infected with a maximum yield of 3.9 $\log_{10}$ $TCID_{50}$/ml in culture supernatant [22]. This low yield slows down the production of PCV2 vaccines. Therefore, increasing the replication of PCV2 in PK-15 cells will allow vaccine production to be scaled up, making the production more efficient and profitable.

Earlier studies have shown that the number of PCV2-infected cells can be enhanced by treatment of PK-15 cells either with interferon-gamma (IFN-γ) [23] or inhibitors of endosomal-lysosomal system acidification such as ammonium chloride (NH_4Cl), chloroquine diphosphate (CQ) and monensin [25]. In the study by Meerts et al. [23], the effect of IFN-γ treatment of PK-15 cells on the PCV2 yield was also investigated, and it was shown that IFN-γ treatment increased the yield of progeny PCV2 by 20 times. Up till now, neither the effect of treatment of PK-15 cells with the endosomal-lysosomal system acidification inhibitors alone nor the effect of treatment of PK-15 cells with the combination of IFN-γ and inhibitors of the endosomal-lysosomal system acidification on PCV2 yield have been investigated. It was the aim of the present study to investigate the combined effect of treating PK-15 cells with IFN-γ and inhibitors of endosomal-lysosomal system acidification on PCV2 infection and PCV2 yield.

To investigate the effect of IFN-γ and inhibitors of the endosomal-lysosomal system acidification on the number of PCV2-infected cells, 2×10^4 PK-15 cells that were free of porcine circoviruses were seeded per well of a 96-well cell culture plate (Nunc, Roskilde, Denmark). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator in culture medium containing 10% fetal bovine serum (FBS; Invitrogen, Grand Island, U.S.A.), 0.3 mg/ml L-glutamine (BDH Chemicals Ltd., Poole, England), 1% nonessential amino acids (100×; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin in RPMI-1640 (Invitrogen). Recombinant porcine IFN-γ (R & D systems, Abingdon, U.K.) was dissolved and stored as previously described [23]. Cell culture medium with or without 500 U/ml IFN-γ was added to PK-15 cells at 6 h post-seeding. At 24 h post-seeding, cells were washed and inoculated with prototype PCV2 strain Stoon-1010 (5.3 $\log_{10}$ $TCID_{50}$, 20th passage) at a

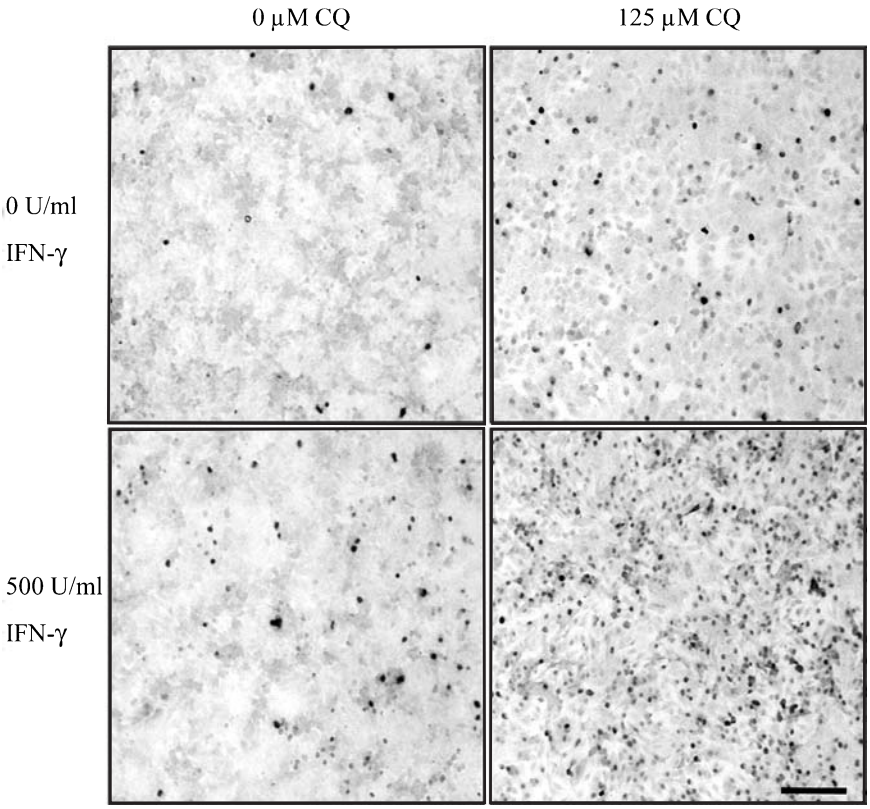

Fig. 1. Effect of IFN-γ and inhibitors of endosomal-lysosomal system acidification on PCV2 infection of PK-15 cells. Representative light microscopic images of PK-15 cells treated with or without IFN-γ and/or CQ after performing an IPMA staining to identify PCV2-infected cells. Bar: 200 μm

multiplicity of infection (moi) of 0.3 at 37 °C. After 1 h, the viral inoculum was washed off and cells were incubated in cell culture medium with or without 25 mM, 125 μM or 6 μM of NH_4Cl, CQ or monensin, respectively. At 24 h post-inoculation (hpi), cell culture medium with or without the endosomal-lysosomal system inhibitors was replaced with fresh cell culture medium without inhibitors of endosomal-lysosomal system acidification. Cells were fixed with methanol at −20 °C for 10 min after the first cycle of PCV2 replication at 36 hpi [22]. PCV2-infected cells were identified by an immunoperoxidase monolayer assay (IPMA) using porcine polyclonal anti-PCV2 antibodies [34] and horseradish peroxidase-conjugated polyclonal rabbit anti-swine immunoglobulins (DakoCytomation, Glostrup, Denmark). Substrate was added to stain-infected cells, which were then counted by examination under a light microscope (Olympus Optical Co., Hamburg, Germany). The number of infected cells per well in untreated PK-15 cells was used as a reference, and all results were expressed as relative percentages to this reference. All experiments were performed three times with each condition in a single experiment in quadruplicate.

Treatment of PK-15 cells either with IFN-γ or inhibitors of endosomal-lysosomal system acidification increased the number of PCV2-infected cells (Figs. 1 and 2), in agreement with previous studies [23, 25]. Combined treatment of PK-15 cells with IFN-γ and inhibitors of endosomal-lysosomal system acidification also increased the number of PCV2-infected cells. The percentages of PCV2-infected PK-15 cells at 36 hpi in untreated and treated PK-15 cells are shown in Table 1. From these results, it can be concluded that inhibiting endosomal-lysosomal system acidification gives an additional enhancement of PCV2 infection in PK-15 cells treated with IFN-γ. This additional effect was possible because IFN-γ and inhibitors of endoso-

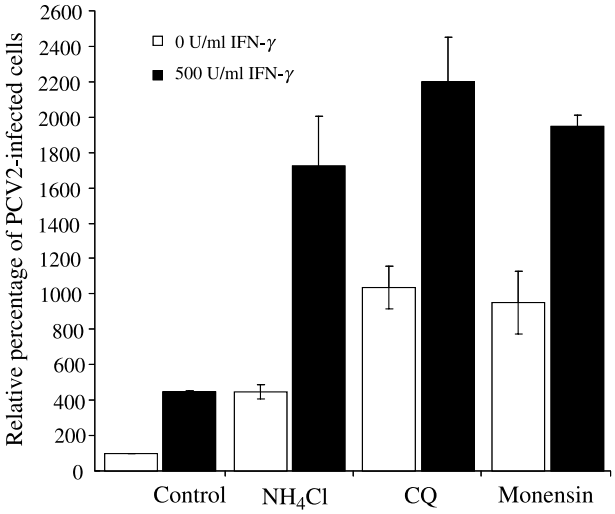

Fig. 2. The effect of IFN-γ and inhibitors of endosomal-lysosomal system acidification on the number of PCV2-infected PK-15 cells. PK-15 cells were pre-treated with (*filled bars*) or without (*empty bars*) IFN-γ before they were inoculated with PCV2. After PCV2 inoculation, cells were incubated with or without inhibitors of endosomal-lysosomal system acidification for 24 h. Cells were fixed at 36 hpi and PCV2-infected cells were counted following IPMA staining. The number of PCV2-infected cells in untreated cell cultures was taken as reference, and all values are relative percentages of this reference

Table 1. Total PCV2 yield in untreated PK-15 cells and PK-15 cells treated with IFN-γ, inhibitors of endosomal-lysosomal system acidification, or a combination of IFN-γ and inhibitors of endosomal-lysosomal system acidification

Lysosomotropic agent	IFN-γ (U/ml)	% of PCV2-infected cells at 36 hpi	PCV2 titre $\log_{10}$ ($TCID_{50}$ per 10^5 cells)			
			1 hpi	24 hpi	48 hpi	72 hpi
Control	0	0.38 ± 0.03	1.9	1.5	2.9	3.1
Control	500	1.71 ± 0.12	2.0	2.1	3.4	3.9
NH_4Cl	0	1.70 ± 0.06	2.1	1.9	3.0	3.8
NH_4Cl	500	6.67 ± 1.57	1.6	3.0	4.0	4.8
CQ	0	3.97 ± 0.31	1.4	1.8	3.3	4.3
CQ	500	8.47 ± 1.23	1.6	2.3	4.0	4.6
Monensin	0	3.61 ± 0.39	1.9	2.3	3.9	3.6
Monensin	500	7.52 ± 1.17	2.0	2.8	4.6	4.8

mal-lysosomal system acidification are known to affect different stages of PCV2 infection. Previous studies have shown that treatment of cells with IFN-γ increases PCV2 infection by increasing internalization of PCV2 particles into the cell [23], while treatment of cells with inhibitors of the endosomal-lysosomal system acidification increases PCV2 infection by increasing disassembly of internalized PCV2 particles within the cell [25].

For virus yield assays, 2×10^5 cells were seeded per well of a 24-well cell culture plate (Nunc). Cells were pre-treated with or without 500 U/ml IFN-γ at 6 h post-seeding. At 24 h post-seeding, cells were washed and inoculated with the prototype PCV2 strain Stoon-1010 (5.3 $\log_{10}$ $TCID_{50}$, 20^{th} passage) at an moi of 0.3 for 1 h at 37 °C. The viral inoculum was washed off, and cells were further incubated in culture medium with or without 25 mM NH_4Cl, 125 μM CQ or 6 μM monensin for 24 h. Then, the culture medium with or without endosomal-lysosomal system acidification inhibitors was replaced with fresh culture medium without inhibitors of endosomal-lysosomal system acidification. All inhibitors of endosomal-lysosomal system acidification were removed at 24 hpi because one of the inhibitors, monensin, is a classical exocytosis blocker [8, 10, 26, 36]. At 1, 24, 48 and 72 hpi, the cell culture supernatant was collected. Cells were subjected to three freeze-thaw cycles. Total virus yield (intra- and extracellular virus titres) was determined on PCV2-negative PK-15 cells by inoculation of a tenfold dilution series on PK-15 cells in quadruplicate, as previously described [24]. After 36 h of cultivation at 37 °C in the presence of 5% CO_2, cells were fixed in methanol at −20 °C for 10 min. Viral antigens were detected using an IPMA as described above, and PCV2 titres were expressed as $\log_{10}$ $TCID_{50}$ per 10^5 cells.

Treatment of PK-15 cells either with IFN-γ or inhibitors of endosomal-lysosomal system acidification increased PCV2 yield (Table 1). Combined treatment of PK-15 cells with IFN-γ and inhibitors of endosomal-lysosomal system acidification further increased PCV2 yield. PCV2 yield increased gradually under all conditions, with the highest yield observed at 72 hpi. The highest recorded PCV2 yield was 4.8 $\log_{10}$ $TCID_{50}$ per 10^5 treated cells compared to 3.1 $\log_{10}$ $TCID_{50}$ per 10^5 untreated cells at 72 hpi. This is a 50-fold increase in PCV2 yield. The highest increase in PCV2 infection was observed when PK-15 cells were treated with a combination of IFN-γ and CQ or NH_4Cl.

The results of the present study show that a combined inhibition of endosomal-lysosomal system acidification and IFN-γ treatment of PK-15 cells increases the number of PCV2-infected cells and PCV2 yield. Treatment of cells with a combination of inhibitors of endosomal-lysosomal system acidification and IFN-γ may be of use in increasing the yield of PCV2 for PCV2 vaccine production.

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